CsrA Regulates Translation of the *Escherichia coli* Carbon Starvation Gene, *cstA*, by Blocking Ribosome Access to the *cstA* Transcript

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Eubacteria are able to rapidly sense, respond to, and adapt to environmental changes such as nutrient availability. Nutrient limitation in *Escherichia coli* can lead to the transition from exponential- to stationary-phase growth. Through the action of global regulatory molecules, such as the stationary-phase sigma factor (σ^S^) encoded by *rpoS*, the induction of numerous *E. coli* genes assists in stationary-phase cell survival and the development of stress resistance (19, 25, 31, 34). The global carbon storage regulatory system (Csr) is involved in the repression of several stationary-phase processes and in the activation of some exponential-phase functions (reviewed in reference 38). Three major components of Csr include RNA binding protein CsrA and two small untranslated RNA molecules, CsrB and CsrC. CsrA represses gluconegenesis, glycogen biosynthesis and catabolism, and biofilm formation (23, 40, 41, 51). Conversely, CsrA activates glycolysis, acetate metabolism, and flagellum biosynthesis (41, 47, 48). Both the CsrB and CsrC RNAs function as antagonists of CsrA by sequestering this protein and preventing its action (20, 29, 49). These regulatory RNAs possess similar imperfect repeat sequences (18 in CsrB and 9 in CsrC) that likely serve as CsrA binding sites (29, 49). Previous studies showed that CsrA, CsrB RNA, and CsrC RNA levels accumulate as the culture reaches the stationary phase of growth (20, 49). CsrA activates transcription of *csrB* and *csrC* indirectly through the BarA/UvrY two-component signal transduction system (36, 45, 49). The purified UvrY protein was found to stimulate expression of *csrB-lacZ* and *csrC-lacZ* transcriptional fusions, indicating that UvrY resides immediately upstream from *csrB* and *csrC* in the signaling pathway (45, 49).

CsrA regulates expression of the glycogen biosynthetic gene *glgC* by binding to two positions in the untranslated leader of the *glgCAP* operon transcript (4). One of the two binding sites overlaps the *glgC* Shine-Dalgarano sequence, while the other CsrA target is located 25 nucleotides (nt) upstream from the Shine-Dalgarano sequence within a short hairpin. CsrA binding to these sites regulates GlgC synthesis by preventing ribosome binding to *cstA* transcripts. RNA footprint and boundary analyses revealed three or four CsrA binding sites, one of which overlaps the *cstA* Shine-Dalgarano sequence, as predicted. These results establish that CsrA regulates translation of *cstA* by sterically interfering with ribosome binding.

CsrA is a global regulator that binds to two sites in the *glgCAP* leader transcript, thereby blocking ribosome access to the *glgC* Shine-Dalgarano sequence. The upstream CsrA binding site (GCACACGGAU) was used to search the *Escherichia coli* genomic sequence for other genes that might be regulated by CsrA. *cstA* contained an exact match that overlapped its Shine-Dalgarano sequence. *cstA* was previously shown to be induced by carbon starvation and to encode a peptide transporter. Expression of a *cstA*-*lacZ* translational fusion in wild-type and *csrA* mutant strains was examined. Expression levels in the *csrA* mutant were approximately twofold higher when cells were grown in Luria broth (LB) and 5- to 10-fold higher when LB was supplemented with glucose. It was previously shown that *cstA* is regulated by the cyclic AMP (cAMP)-cAMP receptor protein complex and transcribed by Eor^S^ (49). We investigated the influence of σ^S^ on *cstA* expression and found that a σ^S^ deficiency resulted in a threefold increase in *cstA* expression in wild-type and *csrA* mutant strains; however, CsrA-dependent regulation was retained. The mechanism of CsrA-mediated *cstA* regulation was also examined in vitro. Cross-linking studies demonstrated that CsrA is a homodimer. Gel mobility shift results showed that CsrA binds specifically to *cstA* RNA, while coupled-transcription-translation and toeprint studies demonstrated that CsrA regulates CstA synthesis by inhibiting ribosome binding to *cstA* transcripts. RNA footprint and boundary analyses revealed three or four CsrA binding sites, one of which overlaps the *cstA* Shine-Dalgarano sequence, as predicted. These results establish that CsrA regulates translation of *cstA* by sterically interfering with ribosome binding.
activity when the medium was depleted of succinate, glycerol, or glucose or when cells grown in Luria broth (LB) entered the stationary phase (42). The cstA promoter contains a putative cAMP receptor protein (CRP) binding site located approximately 80 nt upstream from the start of transcription (42). It was previously shown that cstA expression was dependent on EnvT and the cAMP-CRP complex. All basal and starvation-induced cstA expression was lost in both Δgab and Δgab mutants, while the addition of antibodies specific for EnvT to an in vitro coupled-transcription-translation system eliminated expression (7). The complete CstA polypeptide consists of 701 amino acids and has a hydrophobicity profile indicative of an integral membrane protein (SWISS-PROT accession no. P15078). Results from previous studies led to the conclusion that CstA is involved in peptide transport that would assist the cell in escaping carbon starvation (42). Interestingly, the Pfam database indicates that CstA is conserved in 20 eubacterial and 2 archaebacterial species (www.sanger.ac.uk/cgi-bin/Pfam). Results from the in vivo and in vitro studies reported here establish that CsrA regulates expression of cstA by blocking ribosome access to the cstA Shine-Dalgarno sequence.

MATERIALS AND METHODS

Bacterial strains and plasmids. All of the E. coli strains used in this study are listed in Table 1. The plasmids pTZ18U (United States Biochemical Corp.) and pKK223-3 (Pharmacia) contain a T7 RNA polymerase promoter and a tac promoter, respectively. The plasmid pMLB1034, used for constructing lacZ translational fusions, has been described (44). Plasmid pRRP contains the 39-nt leader, and the first 64 nt of the coding region (1 to +103 relative to the start of lacZ transcription) cloned into the pTZ18U polylinker. This plasmid was used as a template to generate all of the transcripts used in our in vitro studies. pCSB36 contains a cstA-lacZ translational fusion that includes the cstA promoter, the leader, and the first 18 codons of the lacZ coding region (−161 to +92 relative to the start of transcription) cloned into pMLB1034. Plasmid pCSB33 contains a cstA-lacZ translational fusion driven by the tac promoter in pKK223-3. The cstA sequences included in pCSB33 extend from −3 to +95 relative to the start of transcription. E. coli strains CF7789 [ΔlacI lacZ (MluI)] and TR1-SCF7789 [ΔlacZ (MluI) ctsA lacZ (MluI)] were transformed with plasmID3 to generate strains PLB703 and PLB704, respectively. The construction of strains containing single-copy gene insertions of the cstA-lacZ fusion from pCSB33 or pCSB6 followed a published procedure (9). Strains PLB714 and PLB715 contain single-copy cstA-lacZ translational fusions driven by the tac promoters in strains CF7789 and TR1-SCF7789, respectively. Strains PLB726 and PLB727 were constructed by FlpI transductions. Strain PLB726 [ΔlacZ (MluI) ctsA lacZ pcos: Tn10 (Hyb)] was generated by using CAG4513 (polS: Tn10 Tc) as the donor and PLB716 as the recipient strain. Strain PLB727 [ΔlacI lacZ (MluI) ctsA lacZ pcos: Tn10 (Hyb)] was generated by using CAG4513 as the donor and PLB717 as the recipient strain.

Growth studies and β-galactosidase assays. Bacterial growth at 37°C in LB or LB supplemented with 0.2% glucose was monitored with a Klett-Summerson colorimeter (no. 52 green filter). Culture samples (4 ml) were harvested at various times, washed once with 10 mM Tris-HCl, pH 7.5, and frozen as cell pellets at −80°C. Cell extracts were prepared by suspending frozen cell pellets in 0.35 ml of BugBuster protein extraction reagent (Novagen) and incubating them at 37°C in an air shaker. After 1 h, 0.3 ml of Z buffer (33) containing 0.2 mg of lysozyme per ml was added to each sample and incubation was continued for 30 min at 37°C in an air shaker. Cell debris was removed by centrifugation at 4°C. β-Galactosidase assays were performed as described previously (4, 33) with a Packard SpectraCount plate reader to determine absorbance at 420 nm. The specific β-galactosidase activity was determined. Protein concentrations were determined by the Bio-Rad protein assay. CsrA cross-linking. CsrA was purified as previously described (4). Cross-linking experiments with glutaraldehyde were performed by following a previously published procedure (3). Reaction mixtures (final volume, 30 μl) contained 5 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 50 mM KCl, and 0.75% glutaraldehyde. Cross-linking reactions were carried out at 25°C and were started by the addition of 1.4 μg of purified CsrA. After various times one 15-μl aliquot from each reaction mixture was removed and mixed with 15 μl of 2× loading buffer (125 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate, 20% glycerol, 0.02% bromophenol blue). This step was omitted for the other 15-μl aliquot of each reaction mixture. All samples were then immediately heated to 100°C for 5 min. Control samples without glutaraldehyde were treated identically. All samples in loading buffer were fractionated through 4% to 20% gradient denaturing polyacrylamide gels. Protein bands were visualized with Brilliant Blue G colloidal stain (Sigma). The samples not treated with sodium dodecyl sulfate were frozen at −35°C until analyzed by matrix-assisted laser desorption ionization (MALDI-TOF) mass spectrometry.

MALDI-TOF mass spectrometry. Mass spectra of glutaraldehyde-treated and control protein samples were generated with a Voyager-DE STR MALDI-time of flight mass spectrometer (Perseptive Biosystems, Framingham, Mass.). Aliquots from the cross-linking reactions were removed at different reaction times and frozen at −35°C until MALDI-TOF mass spectrometry analysis. Mass spectra were generated using alpha-cyano-4-hydroxycinnamic acid as the matrix. Sample aliquots were cocryostated with the matrix on the MALDI-TOF sample plate at various dilutions, and the dried residues were washed twice with cold 0.1% aqueous trifluoroacetic acid to remove salts and residual glutaraldehyde. A volume of 0.5 μl of 0.1% aqueous octylglucoside was added to each residue, followed by evaporation under ambient laboratory conditions (46), as this was found to improve the mass spectra.

In vitro transcription-translation. In vitro coupled-transcription-translation experiments followed previously published procedures (27, 39), except that final reaction volumes were each 28 μl. The effect of the purified CsrA protein on ctsA-lacZ expression from plasmID3 was examined by using S-30 extracts prepared from strain TR1-5BW3414 (ctsA: Kan’ polS) (27). Proteins synthesized in the reactions were labeled by incorporation of [35S]methionine (1,175 Ci/mmol; NEN Life Science Products) and denatured, and equal volumes of all reaction mixtures were fractionated through 9.5% denaturing polyacrylamide gels. Radiolabeled proteins were detected by fluorography using sodium salicylate (10). Modifications to this assay are described below and in the legend for Fig. 3.

### Table 1. E. coli strains used in this study

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<tr>
<th>Strain</th>
<th>Description</th>
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Gel mobility shift assays. Quantitative gel mobility shift assays followed previously published procedures (4, 50). RNA was synthesized in vitro with the Ambion MEGAscript kit and plasmid pPP1, which had been linearized with BamHI as the template. Gel-purified RNA was dephosphorylated with calf intestinal alkaline phosphatase and subsequently 5' end labeled by using γ-32P-ATP and polynucleotide kinase as described previously (50). Labeled RNA was gel purified, suspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and renatured by being heated to 85°C and slowly cooled at room temperature. Binding reaction mixtures (10 μl) contained 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl2, 32.5 ng of yeast RNA, 10% glycerol, 20 mM dithiothreitol, previously 4 U of RNase inhibitor (Ambion), 0.5 mM 5' end labeled csta RNA, various concentrations of purified CsrA, and 0.1 mg of xylene cyanol/ml. Competition assay mixtures also contained unlabeled RNA competitor (see Results for details). Reaction mixtures were incubated at 37°C for 30 min to allow CsrA-cstA RNA complex formation. Samples were then fractionated on native 12% polyacrylamide gels. Radioactive bands were visualized with a phosphorimager (Molecular Dynamics). Free and bound RNA species were quantified with ImageQuant software (Molecular Dynamics), and the apparent equilibrium binding constant (Kd) and the cooperativity coefficient (n) for CsrA-cstA RNA complex formation were calculated as described previously (50).

Toeprint assay. Toeprint assays were carried out by modifying published procedures (4, 13, 15, 21). cstA transcripts used in this analysis were synthesized with HiDi-purified csta RNA (200 nM) in TE were renatured and hybridized to a DNA oligonucleotide (200 nM) that was complementary to the 3' end of the transcript and that had been labeled at the 5' end with 32P by heating to 80°C followed by slow cooling at room temperature. Toeprint assays were carried out with 1 μM CsrA and/or 100 nM 30S ribosomal subunits and 50 nM RNA(3P). Toeprint reaction mixtures (20 μl) contained 2 μl of the hybridization mixture, 375 μM each dNTP, dithiothreitol, and 10 mM dithiothreitol in toeprint buffer (10 mM Tris-HCl [pH 7.4], 10 mM MgCl2, 60 mM ammonium acetate, 6 mM 2-mercaptoethanol) (21). The reaction mixtures containing CsrA were incubated for 30 min at 37°C to allow CsrA-RNA complex formation. 30S ribosomal subunit toeprint reactions were carried out by incubating RNA, 30S ribosomal subunits, and RNA(3P) in toeprint buffer as described previously (21). Following the addition of 0.6 U of avian myeloblastosis virus reverse transcriptase (Roche) the reaction mixture was further incubated for 15 min at 37°C. Reactions were terminated by adding 12 μl of stop solution (85% formamide, 68 mM EDTA, 0.1× Tris-borate-EDTA, 0.1% xylene cyanol, 0.025% bromophenol blue). Samples were fractionated through a 6% sequencing gel. Sequencing reactions were carried out using pPP1 as the template and the same end-labeled oligonucleotide as a primer.

RNA footprint and structure mapping assays. 5' end-labeled csta transcripts were generated as described for the gel shift assays. Labeled transcripts were used to generate competition RNA samples.

RESULTS
CsrA is a dimer of identical subunits. Initial studies with purified CsrA indicated that the protein existed as either a dimer or trimer in solution (4). Cross-linking experiments were performed to determine which of these two possibilities was correct. Denaturing gel electrophoresis of glutaraldehyde-cross-linked CsrA showed the appearance of a single cross-linked species with the apparent mobility of a dimer (Fig. 1A). MALDI-TOF mass spectrometry of glutaraldehyde-cross-linked CsrA confirmed that CsrA exists as a dimer of identical subunits (Fig. 1B). A minimal signal corresponding to a non-covalent dimer was observed in protein control mass spectra, and a cross-linked dimer was clearly evident in spectra obtained after both 60- and 120-min incubations. No higher oligomers were observed in any MALDI-TOF spectra. Widths of the MALDI-TOF peaks suggest some minor microheterogeneity in both treated and control proteins. All of the molar concentrations used in this study are therefore based on the molecular mass of CsrA as a dimer (13,710 Da).

Identification of csta as a potential CsrA-regulated gene. A sequence comparison of known and putative CsrA binding sites from E. coli, Pseudomonas fluorescens, and Pseudomonas
was recently published (4). The three putative CsrA binding sites from *P. fluorescens* and *P. aeruginosa* overlapped the Shine-Dalgarno sequences of genes involved in the biosynthesis of hydrogen cyanide or exoprotease production (8). The DNA pattern search program available on the Colibri website (http://genolist.pasteur.fr/Colibri/) was used to search for other genes containing the GCACACGGAU sequence found in the upstream CsrA binding site in the *glgCAP* leader transcript. An exact match was identified in *cstA*, a carbon starvation-induced gene that encodes a putative peptide transporter. Interestingly, this sequence overlapped the *cstA* Shine-Dalgarno sequence. The position of this putative CsrA binding site led us to investigate *cstA* as a possible target for posttranscriptional regulation by CsrA.

**CsrA regulates expression of *cstA***. CsrA-dependent regulation of *cstA* was examined in vivo by using a *cstA*-lacZ translational fusion. The fusion included the CRP binding site and the three transcription start sites previously described (42). Expression of the fusion, either from a low-copy-number plasmid (Fig. 2A) or as single-copy chromosomal integrates (Fig. 2B), was monitored. The effect of *csrA* on expression of the *cstA*-lacZ fusion carried on a low-copy-number plasmid when cells were grown in LB is shown in Fig. 2A. As expected, deletion of *csrA* led to a decrease in *cstA* expression, which is consistent with the hypothesis that CsrA functions as a repressor of *cstA* expression.

The effect of *csrA* on expression of the *cstA*-lacZ fusion as a single-copy chromosomal integrate when cells were grown in LB is shown in Fig. 2B. As in the case of the low-copy-number plasmid, deletion of *csrA* led to a decrease in *cstA* expression. However, the level of expression was lower than that observed in the presence of the low-copy-number plasmid. This suggests that the chromosomal integration of the *cstA*-lacZ fusion may have altered the expression of *cstA* in some way.

The effect of *csrA* on expression of the *cstA*-lacZ fusion as a single-copy chromosomal integrate when cells were grown in LB supplemented with 0.2% glucose is shown in Fig. 2C. As in the case of the low-copy-number plasmid and the single-copy chromosomal integrate when cells were grown in LB, deletion of *csrA* led to a decrease in *cstA* expression. However, the level of expression was higher than that observed in the absence of glucose. This suggests that the presence of glucose may have activated the expression of *cstA*.

The effect of *rpoS* on expression of the *cstA*-lacZ fusion as a single-copy chromosomal integrate when cells were grown in LB is shown in Fig. 2D. As expected, deletion of *rpoS* led to an increase in *cstA* expression. This is consistent with the hypothesis that RpoS functions as an activator of *cstA* expression.

The effect of replacing the cAMP-CRP-dependent *cstA* promoter with a tac promoter on CsrA-mediated regulation of the *cstA*-lacZ fusion as a single-copy chromosomal integrate when cells were grown in LB supplemented with 0.2% glucose is shown in Fig. 2E. As expected, replacement of the *cstA* promoter with a tac promoter led to a decrease in *cstA* expression. This is consistent with the hypothesis that the CsrA repressor binds to the CsrA binding site in the *cstA* promoter and inhibits the expression of *cstA*.

The effect of replacing the cAMP-CRP-dependent *cstA* promoter with a tac promoter on CsrA-mediated regulation of the *cstA*-lacZ fusion as a single-copy chromosomal integrate when cells were grown in LB supplemented with 0.2% glucose is shown in Fig. 2F. As expected, replacement of the *cstA* promoter with a tac promoter led to a decrease in *cstA* expression. This is consistent with the hypothesis that the CsrA repressor binds to the CsrA binding site in the *cstA* promoter and inhibits the expression of *cstA*.

These experiments were conducted three times with similar results. Results from representative experiments are shown.

**FIG. 2.** Effect of *csrA* and *rpoS* on expression of a *cstA*-lacZ translational fusion. (A) Effect of *csrA* on expression of the *cstA*-lacZ fusion carried on a low-copy-number plasmid when cells were grown in LB. (B) Effect of *csrA* on expression of the *cstA*-lacZ fusion as a single-copy chromosomal integrate when cells were grown in LB. (C) Effect of *csrA* on expression of the *cstA*-lacZ fusion as a single-copy chromosomal integrate when cells were grown in LB supplemented with 0.2% glucose. (D) Effect of *rpoS* on expression of the *cstA*-lacZ fusion as a single-copy chromosomal integrate when cells were grown in LB. (E) Effect of *rpoS* on expression of the *cstA*-lacZ fusion as a single-copy chromosomal integrate when cells were grown in LB supplemented with 0.2% glucose. (F) Effect of replacing the cAMP-CRP-dependent *cstA* promoter with a tac promoter on CsrA-mediated regulation of the *cstA*-lacZ fusion as a single-copy chromosomal integrate when cells were grown in LB supplemented with 0.2% glucose. Symbols for β-galactosidase activity: wild type, solid squares; *csrA* strain, open squares; *rpoS* strain, solid triangles; *csrA rpoS* strain, open triangles. Symbols for growth: wild type, solid diamonds. Time is hours of cell growth. These experiments were conducted three times with similar results. Results from representative experiments are shown.
2B and C), was examined throughout the growth curve in both wild-type and csrA mutant backgrounds. When the csrA mutant was grown in LB, a reproducible 1.5- to 2-fold increase in expression in was observed regardless of the copy number of the fusion, although expression was about 10-fold higher when the fusion was carried on the low-copy-number plasmid (Fig. 2A and B). CsrA-dependent regulation was most evident during the transition from exponential- to stationary-phase growth and during early stationary phase. Since cstA is involved in nutrient scavenging under conditions of carbon limitation, we hypothesized that the extent of CsrA-dependent repression would be higher under conditions of carbon excess. Thus, cstA expression was also examined when LB was supplemented with 0.2% glucose. Since cstA expression is dependent on cAMP-CRP (42, 43), lower expression was observed throughout the growth curve in both the wild-type and csrA mutant strains under this growth condition. However, expression in the csrA mutant during exponential- and early-stationary-phase growth was 5- to 10-fold higher than expression in the wild-type strain (Fig. 2C).

The dependence of cstA expression on σ70 was previously examined by using an in vitro coupled transcription-translation system (7). The addition of an anti-σ70 antibody eliminated cstA expression. While these results suggested that the strongest promoter(s) for cstA was σ70 dependent, they could not rule out the possibility that the stress response sigma factor, σE, also promoted transcription of cstA. Expression of the integrated cstA-lacZ translational fusion in isogenic wild-type (PLB716), csrA (PLB717), rpoS (PLB726), and rpoS csrA (PLB729) strains grown in LB was examined to test this possibility. The absence of σE resulted in a 2.5- to 3-fold increase in expression. Compare expression levels in PLB726 with those in PLB716 and compare those in PLB729 with those in PLB717 (Fig. 2D). Expression of the cstA-lacZ fusion was also examined when these four strains were grown in LB containing 0.2% glucose. In this growth medium expression in the rpoS strain was fourfold higher than that in the wild-type strain, while expression in the csrA rpoS strain was threefold higher than that in the strain with mutant csrA only (Fig. 2E). These results indicate that Erp does not contribute to cstA transcription. It is important that the CsrA-dependent regulation of cstA expression was maintained in the rpoS mutants. Compare expression in PLB726 with that in PLB729 (Fig. 2D and E). cstA expression in relA and relA spoT mutants was also examined. Expression from both of these strains did not deviate from wild-type levels (data not shown). Thus, it is apparent that expression of cstA is not influenced by ppGpp and the stringent response.

We replaced the native cstA promoter with a tac promoter that does not contain a CRP binding site to examine CsrA-mediated regulation of cstA expression in the absence of the cAMP-CRP effect on transcription (Fig. 2F). Expression from the tac promoter was considerably higher than that from the native promoter in LB medium containing 0.2% glucose (Fig. 2C and F). While the pattern of temporal expression from the tac promoter differed from that from the cstA promoter, CsrA-mediated regulation was retained. Expression from the tac promoter was three- to fourfold higher in the csrA mutant strain than in the wild type.

Since our expression studies indicated that CsrA regulates cstA, coupled-transcription-translation experiments were carried out in S30 extracts prepared from CsrA-deficient cells to determine if purified CsrA could inhibit CstA synthesis (Fig. 3). Transcription of the cstA-lacZ translational fusion containing the native cstA promoter and CRP binding site was dependent on cAMP and CRP, consistent with previously published results demonstrating that cstA expression is subject to catabolite repression (42, 43). Moreover, the addition of purified CsrA inhibited CstA synthesis. As the concentration of CsrA was gradually increased from 0 to 1 μM, we observed a corresponding decrease in the level of the labeled CstA-LacZ fusion polypeptide (Fig. 3). Note that CsrA did not inhibit the expression of other plasmid-carried genes at these concentrations (data not shown).

CsrA binds specifically to cstA RNA. The cstA transcript contains a 39-nt untranslated leader. To characterize the interaction of CsrA with cstA RNA, quantitative gel mobility shift assays were performed with a transcript containing nt +1 to +103 relative to the start of cstA transcription. CsrA binding to this transcript was detected as a distinct band in native gels between 10 and 40 nM CsrA. Complete shifting was observed when the concentration of CsrA reached 80 nM (Fig. 4, top). A nonlinear least-squares analysis of these data yielded an estimated K D value of 40 nM with a cooperativity coefficient (n) of 3.5. The positive cooperativity suggested that the binding of CsrA to one binding site stimulated CsrA interaction with additional binding sites. As the concentration of CsrA was increased further, additional shifted species of higher molecular weights were observed. This gel shift pattern suggested that multiple CsrA molecules were bound to each cstA transcript at these higher CsrA concentrations. Although the stoichiometry of these complexes has not been examined, we presume that the first shifted species contained one cstA transcript and one CsrA dimer and that the additional shifted species contained multiple CsrA molecules. It is possible that the slower-migrating species result from multiple CsrA-RNA and/or CsrA-CsrA interactions.

The specificity of the CsrA-cstA RNA interaction was investigated by performing competition experiments with specific...
(cstA and glgC) and nonspecific (Bacillus subtilis trp leader) unlabeled RNA competitors (Fig. 4, bottom). CsrA was previously shown to bind specifically to glgC RNA with an estimated K_d value of 20 nM (4). Unlabeled cstA and glgC RNAs were effective competitors, whereas the B. subtilis trp leader RNA did not compete with the CsrA-cstA RNA interaction. These results establish that CsrA binds specifically to cstA RNA.

CsrA binding to the cstA transcript inhibits CstA synthesis by preventing ribosome binding. A toeprint analysis was performed to identify the position(s) of bound CsrA in the cstA transcript. The presence of bound CsrA should stop primer extension by reverse transcriptase, resulting in a toeprint band near the 3' boundary of CsrA. The results of the toeprint experiments are presented in Fig. 5 and summarized in Fig. 6A. Seven CsrA-dependent toeprint bands were clustered in three segments of the cstA transcript (Fig. 5). Results from footprinting and boundary studies (see below) suggest that the bands at positions C38, U41, A46, A59, and C61 constitute authentic CsrA toeprints. The two bands near the top of the gel (Fig. 5) are located within the first 10 nt of the natural cstA transcript. Since bound CsrA does not protect the cstA transcript upstream of U10 from RNase cleavage (see below), it is unlikely that these bands represent authentic CsrA toeprints. The two prominent bands at the bottom of the gel (Fig. 5) are present in each lane and are not dependent on CsrA. Since RNA secondary structure can inhibit primer extension by reverse transcriptase, these bands appear to be RNA structural toeprints.

Similar toeprint experiments were performed to identify the positions of bound 30S ribosomal subunits. As expected, a prominent tRNA^{Met}-dependent toeprint band was observed 15 nt downstream from the A of the AUG initiation codon (Fig. 5). Toeprint experiments were also carried out to determine if CsrA could inhibit ribosome binding. When CsrA was bound to the cstA transcript prior to the addition of 30S ribosomes and tRNA^{Met}, several of the CsrA-dependent toeprint bands were observed, whereas the ribosome toeprint was considerably reduced (Fig. 5). These results demonstrate that bound CsrA inhibits ribosome binding to the cstA transcript. When taken together with the in vivo expression and coupled-transcription-translation results demonstrating that CsrA regulates cstA expression and CstA synthesis, respectively, the toeprint results establish that CsrA regulates translation initiation of cstA by blocking ribosome access to the cstA ribosome binding site.

Identification of the CsrA binding sites in the cstA transcript. Since the gel shift and toeprint results indicated that multiple molecules of CsrA can bind to the cstA transcript (Fig. 4 and 5), CsrA-cstA RNA footprint experiments were carried out to identify the CsrA binding sites. Three single-strand-specific RNases were used as probes for these studies. The results of the footprint experiments are presented in Fig. 7 and summarized in Fig. 6A. As the concentration of CsrA was increased from 0 to 4 μM, protection of several nucleotides from RNase T1 (G-specific), RNase T2 (A preference), and/or RNase A (C- and U-specific) cleavage was observed (Fig. 7).
secondary structure in this segment of the cstA transcript (Fig. 7).

To more precisely define the CsrA binding sites, we performed a boundary analysis, a technique that is useful for delineating the 3' and 5' boundaries of an RNA binding target. Thus, information derived from a boundary experiment differs from the information obtained from a footprint analysis. Whereas a footprint reveals the nucleotides that are protected from enzymatic or chemical probes, boundary experiments separately reveal the 5' and 3' nucleotides that are required for binding. Thus, the two techniques complement one another. The results of the boundary analysis are presented in Fig. 8 and summarized in Fig. 6A. RNAs were 5' or 3' end labeled, base hydrolyzed, and subsequently incubated in the presence of 1 or 3 μM CsrA. Samples were fractionated through a native gel followed by autoradiography. In each case three diffuse bands, corresponding to CsrA-cstA RNA complexes with different stoichiometries, and a single band for unbound RNA were observed. The RNA from each of these bands was gel purified and subsequently fractionated side by side under denaturing conditions. The cutoffs for the 3' boundary analysis (5'-end-labeled RNA) were relatively sharp. The experiment carried out with 1 μM CsrA revealed 3' boundaries at positions G30, G53, and A70 (Fig. 8). An additional 3' boundary at A19 was observed with 3 μM CsrA, suggesting that CsrA can independently bind to this site only at this higher protein concentration. Thus at a CsrA concentration of 3 μM, transcripts ending between positions 0 and 18 were unbound, while the transcripts ending at positions 19 to 29, 30 to 52, 53 to 69, and 70 to full-length RNA presumably had one, two, three, and four bound CsrA dimers, respectively. Taken together, the 3' boundary results suggest that four CsrA binding sites are present in the cstA transcript (Fig. 6A and 8). The 5' boundary analysis (3'-end-labeled RNA) was largely consistent with the 3' boundary results. With 1 μM CsrA 5' boundaries at A27 and G42 were observed. 5' boundaries were also observed in the presence of 3 μM CsrA at U16, A36, and C50 (Fig. 8). As can be seen in Fig. 6A, paired 5' and 3' boundaries were observed in three cases. In each case, CsrA protected the residues between these boundary pairs from nucleolytic cleavage, indicating that each of these RNA segments is contained within a distinct CsrA binding site. As discussed below, our data suggest that an additional CsrA binding site is present between positions 65 and 75.

**DISCUSSION**

cstA encodes a 701-amino-acid hydrophobic polypeptide (SWISS-PROT accession no. P15078) that appears to be a peptide permease (42). The cstA promoter and the effect of carbon starvation on its expression have been investigated previously (7, 31, 42, 43). The cst genes including cstA are activated by cAMP-CRP. Primer extension studies revealed three potential cstA transcription start sites, with the upstream site being the strongest. Potential Erh +10 to +10 sequences are located upstream from each of these sites. Deletion of an apparent CRP binding target located 80 nt upstream from the 5'-most transcriptional start site eliminated expression from all three of these putative promoters (31). In vitro coupled-transcription-translation studies confirmed that cstA expression was depen-
dent on cAMP and Erh0 (7). Although there was some basal cstA expression during the exponential phase of cells grown in LB, expression was highest after glucose starvation; glucose depletion resulted in sixfold-higher expression (7).

CsrA of *E. coli* is a global regulatory RNA binding protein that controls the expression of genes involved in central carbon metabolism, motility, and biofilm development (23, 38, 48). CsrA binds specifically to the *glgCAP* leader transcript and blocks translation of *glgC* (4). Inhibition of *glgC* translation presumably leads to accelerated *glgCAP* mRNA decay (27, 28, 40). The identification of a potential CsrA binding site (GCA CACGGAU) that overlapped the *cstA* Shine-Dalgarno (SD) sequence led us to investigate CsrA-dependent regulation of this gene. Our genetic analysis of the expression of a *cstA-lacZ* translational fusion demonstrates that expression of *cstA* is repressed by CsrA (Fig. 2). Although the difference in expression levels between wild-type and *csrA* mutant strains was a modest 1.5- to 2-fold when strains were grown in LB (Fig. 2A and B), there was a 5- to 10-fold difference when the same strains were grown in LB supplemented with 0.2% glucose (Fig. 2C). Results from the coupled-transcription-translation experiments demonstrating that CsrA inhibits CstA-LacZ synthesis (Fig. 3) are consistent with the results of the in vivo expression studies.

We also investigated the effect of *rpoS* (σ5) on expression of *cstA* and found that transcription of this gene is σ5 independent. In fact, *cstA* expression increased two- to sixfold in *rpoS* mutant strains, although CsrA-dependent regulation was retained (Fig. 2D and E). In a previous study examining the *lamB* and *mglBAD* genes, which are responsible for the high-affinity pathway of glucose uptake in *E. coli*, it was shown that glucose limitation resulted in higher expression of these transporter genes in an *rpoS* mutant (35). Glucose limitation leads to the hunger response, which results in increased expression of many high-affinity transporter genes that are necessary for survival under low-nutrient conditions (18). It was shown that there is

**FIG. 7.** CsrA-cstA RNA footprint and RNA structure mapping. 5'-end-labeled *cstA* RNA was treated with RNase T1, RNase T2, or RNase A. Reactions were carried out in the absence or presence of CsrA at the concentrations indicated at the top of the lanes. Lanes corresponding to mock-treated RNA (M) and control RNA (C), as well as partial hydrolysis (OH) and RNase T1 digestion (T1) ladders are shown. The RNase T1 ladder was generated under denaturing conditions so that every G residues in the transcript could be visualized. The nucleotides that were protected from RNase cleavage (−), as well as those that were enhanced for cleavage (+), by bound CsrA are shown at the right of each panel. Positions of the *cstA* Shine-Dalgarno (SD) sequence and start codon (Met) are shown. Numbering is from the start of *cstA* transcription.
a selective advantage for bacteria when they lose $\sigma^S$ under nutrient-limiting conditions (52). It was also reported that competition between $\sigma^S$ and other sigma factors leads to reduced expression from non-$\sigma^S$ promoters (16, 52). Our results suggest that expression of $\text{cstA}$ is influenced by competition between $\sigma^S$ and $\sigma^{70}$ by a similar mechanism.

While results from the in vivo studies establish that CsrA regulates $\text{cstA}$ expression (Fig. 2), the basic mechanism of CsrA-mediated control was revealed by in vitro analyses. Note that the $\text{cstA}$ transcript used in these studies contained all three of the putative transcription start sites (discussed above). CsrA is a homodimer (Fig. 1) that binds specifically to the $\text{cstA}$ transcript (Fig. 4), thereby inhibiting ribosome binding (Fig. 5). The finding that CsrA-$\text{cstA}$ RNA complex formation was cooperative suggested that the transcript used in these studies contained multiple CsrA targets. Footprinting and boundary analyses confirmed the presence of three and possibly four CsrA binding sites in the $\text{cstA}$ transcript (Fig. 6 to 8). The gel shift data indicate that interaction of CsrA with the $\text{cstA}$ transcript is highly cooperative. Twofold increases in CsrA concentration resulted in the disappearance of one species and the appearance of a slower-migrating species (Fig. 4). This gel shift pattern is similar to what was previously observed for CsrA interaction with $\text{glgC}$, $\text{csrB}$, and $\text{csrC}$ transcripts (4, 49). This gel shift pattern indicates that multiple CsrA dimers were bound to each $\text{cstA}$ transcript at these higher concentrations. Note that the number of distinct complexes exceeds the number of CsrA binding sites that we identified in the transcript, suggesting that some of the CsrA dimers present in the slower-migrating complexes bind only via protein-protein interactions and not by directly interacting with RNA. Based on the footprinting data, one of the CsrA binding sites (site II) extends from A25 to A33 and overlaps the $\text{cstA}$ Shine-Dalgarno sequence. A second site, which is positioned just upstream (site I), extends from U10 to C22 (Fig. 6). The fact that these two CsrA binding sites are adjacent to one another, combined with the finding that the CsrA-$\text{cstA}$ interaction is cooperative (Fig. 4), suggests that protein-protein contacts likely contribute to CsrA-$\text{cstA}$ RNA complex formation. The third binding site (site III), which extends from C50 to A55, is just downstream from the start of $\text{cstA}$ translation and overlaps the position of the ribosome toeprint (Fig. 6A). Thus, CsrA bound to each of these sites would directly interfere with ribosome binding. In addition to these three sites, there is some evidence for a fourth binding site further downstream (site IV). Residue C71 was protected from RNase cleavage, and a 3' boundary be-
between A70 and C71 was observed. Furthermore, the sequence of this RNA segment is similar to those of other known CsrA targets (see below). However, in this case a corresponding 5’ boundary was not observed. In the 5’ boundary analysis this would be the only remaining binding site in the transcript. Perhaps the affinity of CsrA for this site is relatively weak, and occupancy of this site may require protein–protein contact with another bound CsrA molecule. 5’ boundaries between binding sites II and III were also observed (Fig. 6A). The finding that bound CsrA led to enhanced RNase cleavage in this RNA segment and the absence of corresponding 3’ boundaries suggest that these 5’ boundaries are artifacts. Taken together, our results establish that CsrA binds to three or four sites in the cstA transcript and that bound CsrA inhibits CstA synthesis by sterically interfering with ribosome binding.

Both glgC (glycogen biosynthesis) and cstA (carbon starvation) are involved in carbon metabolism. Interestingly, expression of both genes is activated by CAMP-CRP and repressed by CsrA. Nevertheless, these two regulatory systems do not invariably act antagonistically to regulate gene expression. CsrA and CAMP-CRP both activate motility in E. coli through effects on flhDC expression (48). In addition, the effects of CsrA on glycogen synthesis are mediated independently of cpr and cya (40). CsrA regulates gene expression posttranscriptionally, and its steady-state levels accumulate only moderately at the transition to stationary phase (20). Thus, CsrA regulation generally does not determine the temporal pattern of gene expression but is superimposed on it (40).

CsrA was previously shown to regulate glgC expression by a translation control mechanism that is similar to what was found for cstA; however, one key distinction between the two regulatory mechanisms is that CsrA binding to glgCAP leader RNA is not cooperative (4). Two CsrA binding sites in the glgCAP leader transcript were identified. The downstream site overlaps the glgC Shine-Dalgarno sequence and is single stranded (4). In contrast, the upstream binding site has the potential of being partially sequestered in a short RNA hairpin, a situation that is similar to several of the presumed CsrA binding sites in CsrB RNA (4, 29). However, in each of these binding sites the most highly conserved sequence element (GGA) is located in the loop of the putative hairpin. Furthermore, for glgC it was found that the RNA secondary structure is unstable (4). Since the cstA transcript used in our studies did not have any stable secondary structure, it is likely that CsrA binds preferentially to single-stranded RNA. A sequence alignment of the known (gglC and cstA) and presumed (CsrB and CsrC) CsrA binding sites in E. coli is shown in Fig. 6B. From this alignment it is apparent that the GGA in the center of the consensus YAN GGANR is the most highly conserved element. Interestingly, the ANGGA residues are conserved in the presumed CsrA binding targets in Pseudomonas species that were identified genetically (8).

Csr systems regulate a variety of important genes and processes in several other organisms as well. In addition to hydrogen cyanide production and exoprotease production in Pseudomonas species (8), control of both of the quorum-sensing systems in P. aeruginosa is by CsrA (RsmA) (37). Furthermore, the CsrA homolog in Erwinia species (RsmA) regulates quorum sensing and a variety of genes involved in soft-rot disease of higher plants (11, 12, 24). In addition, CsrA and CsrB regulate epithelial cell invasion by Salmonella enterica (1, 2), and CsrA (RsmA) affects swarming and other activities of Proteus mirabilis and Legionella pneumophila (17, 26). Despite the importance of Csr as a global regulator of bacterial physiology and pathogenesis, the molecular mechanism(s) of CsrA-mediated gene regulation has been elucidated only for glgC and cstA of E. coli. While it appears that the three genes in Pseudomonas species are regulated by translation control mechanisms similar to those for E. coli glgC and cstA, it is clear that CsrA-mediated positive control (e.g., E. coli flhDC) (48) must occur by a different mechanism.

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